

attempted to simplify the method by replacing the deuterium oxide with tritium-enriched water. Tritiated water was added to a stock solution of the protein and samples were withdrawn at timed intervals. The samples were freeze-dried and the last traces of excess THO were removed at elevated temperature. The residual protein was dissolved, one aliquot was used for determination of the protein content, and another aliquot was used for tritium assay in a scintillation counter. The main advantage of this method is the opportunity afforded to start the experiment with a protein already in solution. There is no necessity for the protein to be able to withstand lyophilization without denaturation, since it is unimportant for the result whether the protein denatures in the final lyophilization.

Englander²¹ has modified the tritium technique in such a way that the lyophilization step is completely omitted. After reaction of the protein with tritiated water for a given length of time, the protein is separated from tritiated water with a short Sephadex G-25 column. The use of a short column (3×6 cm) and a high flow rate made possible the isolation of the protein as it emerged from the column after about 2 minutes. It was well separated from the tritiated water. From the specific radioactivity of the protein fraction the exchange-in rate of tritium was calculated. With a small modification the method could be used for measuring the exchange-out of tritium under the same conditions. The simple and elegant Sephadex method has so far been used on only a few proteins, but further results with this technique are awaited with interest.

Systematic errors are introduced by the tritium methods, if the tritium enrichment factor in the protein is different from the tritium enrichment factor in the solvent. Some experiments indicate such equilibrium isotope effects to be very small, but further studies are indicated. If systematic errors of this kind exist, they become of much smaller importance if the method is used to study closely related protein systems. The tritium-Sephadex method might, for instance, be useful for studying the influence of chemical substitutions on protein conformations.

²¹ E. W. Englander, *Biochemistry* **2**, 798 (1963).

[86] Difference Spectroscopy

By T. T. HERSKOVITS

The light absorption of proteins in the ultraviolet (250–300 m μ) region is predominantly due to the electronic excitations of their aromatic amino acid side chains, tyrosine, tryptophan, and to some extent phenylalanine. Since the tyrosine and tryptophan residues in the average

globular protein are relatively few in number, the perturbations in the absorption spectra of these chromophoric residues can be utilized as natural probes to give answers to a number of questions of interest to protein chemists related to the structure of proteins in solution. Information concerning the environment and location of aromatic side chains and the changes in environment of these residues due to structural transformations produced by changes in pH, temperature, solvent composition, etc., can be gained from changes in spectra and difference spectra. Difference spectral methods, to be described, also offer the possibility of dealing experimentally with questions concerning the involvement and location of natural and artificial chromophoric groups in enzyme-substrate and protein-protein interactions.

The Solvent Perturbation Method

Principle. The solvent perturbation technique of difference spectroscopy for studying the location of chromophoric amino acids in proteins is based on the following considerations. The spectra of tyrosine or tryptophan residues located on the surface of proteins are usually perturbed by changes in physical properties of the solvent (e.g., changes in refractive index, dielectric constant, and solvent-solute interactions such as hydrogen bonding,¹⁻⁵ etc.) in the immediate vicinity of these chromophores. On the other hand, tyrosines and tryptophans deeply buried in the interior folds of the protein are shielded from the perturbing effect of the solvent since they do not come freely in contact with it. Consequently their spectra are not affected by the change in solvent properties. In this technique, mild or inert substances, which do not alter the conformation of the protein but are known to produce accurately measurable spectral shifts, are employed as perturbants in low concentrations (10–20%).^{6,7}

In the case of an average globular protein, with some groups buried and some exposed, a given perturbant will produce only a fraction of the total spectral shift that would be given by the fully unfolded protein chain or an amino acid mixture corresponding in composition to the protein. This fraction of the total spectral shift is then a measure of the fraction of chromophoric groups exposed or accessible to solvent.

In practice, many of the experimental problems connected with such

¹ N. S. Bayliss and E. G. McRae, *J. Phys. Chem.* **58**, 1002, 1006 (1954).

² Y. Ooshika, *J. Phys. Soc. Japan* **9**, 594 (1954).

³ E. G. McRae, *J. Phys. Chem.* **61**, 562 (1957).

⁴ H. C. Longuet-Higgins and J. A. Pople, *J. Chem. Phys.* **27**, 192 (1957).

⁵ M. Ito, K. Inuzuka, and S. Imanishi, *J. Am. Chem. Soc.* **82**, 1317 (1960).

⁶ T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.* **235**, PC56 (1960).

⁷ T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.* **237**, 2481 (1962).

measurements are greatly simplified by use of difference spectral techniques. Instead of comparing the spectral shifts of native proteins with the shifts of the proteins in the unfolded state or with those produced by model compounds, which from the experimental point of view is a difficult task, the difference spectra of these substances are obtained and compared.⁸ The technique of difference spectroscopy consists of measuring the absorption of a test solution of protein or model compound against a reference solution of identical concentration. The parameters which can be varied in the test solution are the pH, temperature, electrolyte concentration, or denaturing agent concentration. In the solvent perturbation technique all these conditions are kept constant; only the composition of the solvent is varied by use of such additives as sucrose, glycerol, ethylene glycol, dimethylsulfoxide, etc.

Because these additives usually decrease the polarity of the solvent, they shift the absorption maxima of the aromatic amino acids to longer wavelengths (red shifts). These shifts have been attributed to the difference in energy levels between the ground state and the first excited state of $\pi \rightarrow \pi^*$ electronic transitions.¹¹⁻¹³ Figure 1 depicts the sort of spectral shifts encountered by use of these perturbants, and shows the relation between these solvent-induced shifts and the so-called "solvent perturbation difference spectra." The data presented in Fig. 1 were obtained with *N*-acetyl ethyl esters of tryptophan, tyrosine, and phenylalanine, employing 20% dimethylsulfoxide as the perturbing additive. It should be noted that the two characteristic maxima of tryptophan difference spectra (and the difference spectra of proteins in which the absorption is dominated by tryptophan) appear at 292–294 $m\mu$ and 281–284 $m\mu$. In the case of tyrosine and tyrosine-rich proteins the first maximum appears in the trough region of tryptophan, at 286–288 $m\mu$, while the second maximum is located at 278–281 $m\mu$. It is clear from the differ-

⁸ For chromophoric substances the difference spectrum, for a given wavelength, is proportional to the spectral shift, $\Delta\lambda$. At wavelength λ the molar absorptivity difference, $\Delta\epsilon_\lambda$, is equal to $-(d\epsilon/d\lambda)_\lambda \Delta\lambda$, where $(d\epsilon/d\lambda)_\lambda$ is the slope of the direct spectrum.^{9,10} This approximation holds for small spectral shifts not accompanied by significant changes in shape and amplitude of the direct spectrum. The spectral shifts of proteins due to the usual 20% perturbants employed (see Table II) are of the order of 0.1–1 $m\mu$. These shifts are clearly too small for accurate direct experimental determination.

⁹ C. H. Chervenka, *Biochim. Biophys. Acta* **26**, 222 (1957).

¹⁰ J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.* **83**, 2686 (1961).

¹¹ C. Reid, "Excited States in Chemistry and Biology." Butterworth, London, 1957.

¹² W. Kauzmann, "Quantum Chemistry." Academic Press, New York, 1957.

¹³ D. B. Wetlaufer, *Advan. Protein Chem.* **17**, 303 (1962).

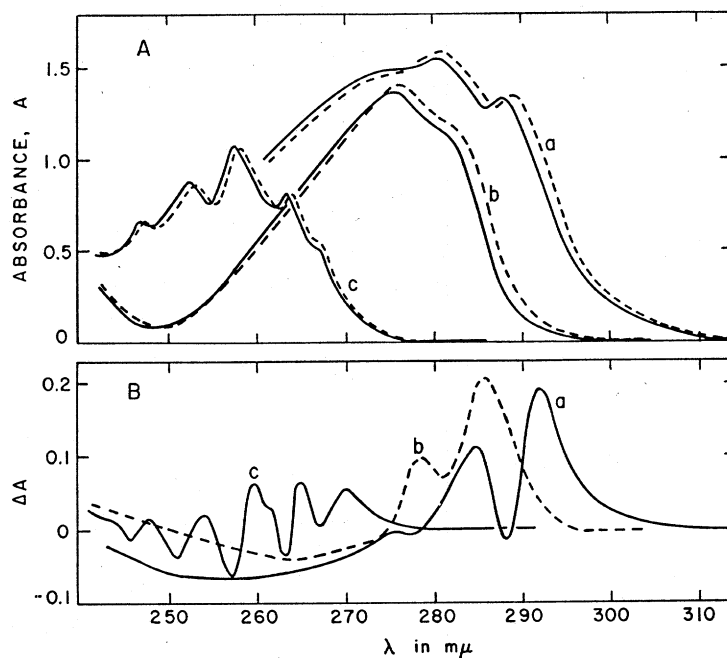


FIG. 1. Relation between spectral shifts and solvent perturbation difference spectra of *N*-acetyl ethyl esters of tryptophan (curves *a*), tyrosine (curves *b*), and phenylalanine (curves *c*): (A) *solid lines*, spectra in water; *broken lines*, spectra in the presence of 20% dimethylsulfoxide; (B) difference spectra obtained with 20% dimethylsulfoxide solutions plus water blanks in the sample beam, and 20% dimethylsulfoxide blank and aqueous solution in the reference beam of the spectrophotometer. Concentrations: $3 \times 10^{-4} M$ *N*-acetyl-L-tryptophan ethyl ester, $1 \times 10^{-3} M$ *N*-acetyl-L-tyrosine ethyl ester, and $5 \times 10^{-3} M$ *N*-acetyl-L-phenylalanine ethyl ester; buffer: $\Gamma/2 = 0.01$, pH 6.8, phosphate [representation of data following S. Yanari and F. A. Bovey, *J. Biol. Chem.* **235**, 2818 (1960)].

ence spectral data of phenylalanine that this amino acid contributes little or nothing in the absorption region of interest, namely, above 270 $m\mu$. Consequently, for all practical purposes the contribution of phenylalanine to the difference spectra of proteins in this wavelength region may be neglected.

In Fig. 2 are shown the difference spectra of two proteins in the native state and in the unfolded state (in 8 *M* urea), together with the difference spectra of model amino acid mixtures corresponding to their aromatic amino acid composition. The difference spectra of serum albumin are characteristic of the perturbation difference spectra of tyrosine-rich proteins, with appropriate maxima at 286–288 and 278–281 $m\mu$. The difference spectra of lysozyme, on the other hand, are primarily

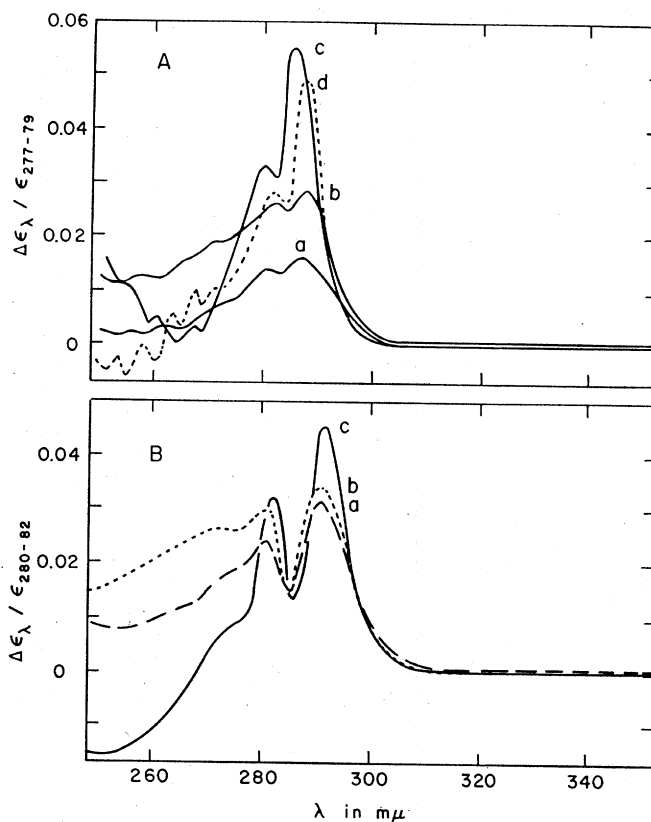


FIG. 2. Solvent perturbation difference spectra of human serum albumin and lysozyme and their respective model compound mixtures obtained with 20% glycerol as perturbant: (A) human serum albumin. Curve *a*, native protein, pH 7.6. Curve *b*, acid-isomerized protein, pH 2.1 (see text under "Application" concerning this). Curve *c*, disulfide-cleaved protein in 8 *M* urea in the presence of 0.02 *M* thioglycolate, pH 4.5. Curve *d*, model compound mixture consisting of 1.5×10^{-3} *M* *N*-acetyl-L-tyrosine ethyl ester, 5×10^{-5} *M* *N*-acetyl-L-tryptophan ethyl ester, and 2.8×10^{-3} *M* phenylalanine ethyl ester, pH 3.5. Protein concentration 0.3%, $\Gamma/2 = 0.25$ [redrawn from T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.* **237**, 2481 (1962)]. (B) lysozyme. Curve *a*, native protein, pH 5.1. Curve *b*, disulfide-cleaved protein in 8 *M* urea, pH 5.1. Curve *c*, model compound mixture consisting of 4×10^{-4} *M* *N*-acetyl-L-tryptophan ethyl ester and 2×10^{-4} *M* *N*-acetyl-L-tyrosine ethyl ester, pH 5.8. Protein concentration, 0.1%; $\Gamma/2 = 0.2$ [data of E. J. Williams, T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.* **240**, 3574 (1965)].

due to tryptophan, and show maxima at 292–294 and 281–284 $m\mu$. It should be noted that the perturbations obtained with denatured, disulfide-cleaved proteins are as a rule greater than those given by the native proteins in water.

Estimation of the Fraction of Exposed Tyrosyl and Tryptophyl Residues. To obtain an estimate of the fraction of exposed chromophoric residues in proteins, suitable reference values for the difference spectral parameters are required. The choice of "100% exposed" reference can be approached in one of two ways: (a) calculations can be based on the parameters obtained with the free amino acids or with a suitable model compound mixture (for example, *N*-acetyl ethyl esters of tyrosine and tryptophan and possibly phenylalanine, shown in Fig. 2), corresponding exactly to the chromophore composition of the protein, or (b) the unfolded protein chain itself can be used as a reference standard, provided there is reasonable assurance that in the denaturation or unfolding process all the chromophores have been fully exposed and are free to come in contact with the solvent. On steric grounds, the use of the unfolded protein chain for the establishment of the difference spectral constant is preferred, since the polypeptide chain itself as well as the adjoining side-chain groups may cause some interference with solvent access.^{6,7} In addition, the amino acid composition of a given protein may sometimes vary, depending on the source and method of protein preparation. Even in the case of well-characterized proteins, the precise tryptophan and even tyrosine content is sometimes in question (this is discussed in this volume [58-61]).

Unfortunately, fully denatured proteins, with all their disulfide bonds cleaved, are rarely soluble in aqueous solutions. For this reason measurements on the disulfide-cleaved proteins have been made in 8 *M* urea solutions.¹⁴⁻¹⁶ Urea has an effect, however, on the difference spectral parameters of tyrosine and tryptophan^{15,16} (see also Table I), and for this reason measurements of the model compound mixtures must be made for each protein in water and 8 *M* urea, and the data obtained on the disulfide-cleaved protein in 8 *M* urea must be corrected for this solvent effect. As shown in Table I, the correction factor may affect the reference parameters by as much as 20%, depending on the type of perturbant employed. Curves *c* and *d* of Fig. 2A and curves *b* and *c* of Fig. 2B present a comparison between the difference spectra of human serum albumin and lysozyme in the unfolded, disulfide-cleaved state and their model amino acid analogs.

To illustrate the method of handling solvent perturbation data, the data of Williams *et al.*,¹⁵ obtained with lysozyme and α -chymotrypsinogen, are presented in Table I (data derived from Fig. 2B are in-

¹⁴ T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.* **237**, 3418 (1962).

¹⁵ E. J. Williams, T. T. Herskovits, and M. Laskowski, Jr., *J. Biol. Chem.* **240**, 3574 (1965).

¹⁶ T. T. Herskovits, *J. Biol. Chem.* **240**, 628 (1965).

TABLE I
SUMMARY OF DIFFERENCE SPECTRAL RESULTS FOR LYSOZYME
AND α -CHYMOTRYPSINOGEN
($\Delta\epsilon_{222}/\epsilon_{282}$)

Perturbant 20% ^a	Native protein in water ^b	Di- sulfide- cleaved protein in 8 <i>M</i> urea ^{b,c}	Model mixture in water ^d	Model mixture in 8 <i>M</i> urea ^d	Fraction of exposed tryptophyls ^e	
					<i>R_P</i>	(<i>R_M</i>)
Lysozyme						
Sucrose	0.021	0.028	0.027	0.027	0.75	(0.77)
Glycerol	0.031	0.035	0.046	0.042	0.79	(0.68)
Ethylene glycol	0.034	0.037	0.049	0.042	0.79	(0.69)
Methanol	0.028	0.040	0.038	0.037	0.68	(0.73)
Dimethylsulfoxide	0.056	0.075	0.088	0.081	0.69	(0.64)
Polyethylene glycol ^f	0.043	0.060	0.088	0.066	0.54	(0.49)
α-Chymotrypsinogen						
Sucrose	0.015	0.031	0.028	0.028	0.49	(0.55)
Glycerol	0.016	0.034	0.045	0.041	0.43	(0.35)
Ethylene glycol	0.017	0.034	0.048	0.042	0.44	(0.35)
Dimethylsulfoxide	0.027	0.063	0.088	0.081	0.39	(0.31)
Polyethylene glycol ^f	0.026	0.047	0.087	0.065	0.41	(0.30)

^a With the exception of sucrose, 20 volumes of liquid perturbants were used per 100 volumes of final solution. Sucrose solutions contained 21.6 g sucrose per 100 ml solution (such a solution *in water* contains 20% sucrose by weight).

^b $\Gamma/2 = 0.1$ – 0.2 , pH 2.5–7.0. Lysozyme concentration, 0.1%; α -chymotrypsinogen concentration, 0.13–0.15%.

^c Disulfide-cleaved lysozyme was reduced (see section on preparation of solutions) in 8 M urea in the presence of 0.02 M thioglycolate. Disulfide-cleaved α -chymotrypsinogen was obtained by *S*-sulfonation [J. F. Pechère, G. H. Dixon, R. H. Maybury, and H. Neurath, *J. Biol. Chem.* **233**, 1364 (1958)]. The protein solution used with 20% sucrose was reduced with thioglycolic acid in 8 M urea.

^d Mixtures of *N*-acetyl ethyl esters of tryptophan and tyrosine. Molar ratio of 6:3 for lysozyme and 7:4 for α -chymotrypsinogen.

^e Fraction of exposed groups estimated relative to disulfide-cleaved protein and corrected for urea contribution to $\Delta\epsilon_{222}/\epsilon_{282}$ (see text concerning this correction); values in parentheses are relative to model analogs in aqueous solution.

^f Degree of polymerization = 6.

cluded). The fraction of exposed chromophoric residues has been calculated by use of the relation

$$R_P = \frac{(\Delta\epsilon_{\lambda,\max}/\epsilon_{\lambda,\max})_{\text{protein}}}{(\Delta\epsilon_{\lambda,\max}/\epsilon_{\lambda,\max})_{\text{unfolded protein, 8 M urea}}} \times F \quad (1)$$

where

$$F = \frac{(\Delta\epsilon_{\lambda,\max}/\epsilon_{\lambda,\max})_{\text{model cpd in 8 M urea}}}{(\Delta\epsilon_{\lambda,\max}/\epsilon_{\lambda,\max})_{\text{model cpd in water}}}$$

Here the difference spectral constant, $\Delta\epsilon_{\lambda,\max}/\epsilon_{\lambda,\max}$, is defined as the ratio of the molar absorptivity difference, $\Delta\epsilon_{\lambda,\max}$, and the molar absorptivity, $\epsilon_{\lambda,\max}$. The subscripts, λ,\max , refer to the wavelength of the first difference spectral maximum (for $\Delta\epsilon$) and the maximum of the direct spectrum of the protein or model amino acid (usually at 275–282 m μ for ϵ). The factor F corrects for the effect of urea on the difference spectral intensities of the chromophores. F has values of about 0.8–1.2, depending on the perturbant and the chromophore. For the two enzymes in Table I, as well as for other proteins the spectra of which are dominated by tryptophan absorption, $\Delta\epsilon_{\lambda,\max}$ refers to the major tryptophan difference peak at 292–294 m μ . The tyrosine content of proteins devoid of tryptophan, or having low tryptophan content, may be estimated from the intensities of the 286–288-m μ tyrosine difference spectral maximum (Fig. 2A). Examples of such proteins are ribonuclease,⁶ insulin,¹⁷ and the serum albumins.⁷

As suggested by earlier comments on model compound mixtures, a somewhat less satisfactory estimate of the fraction of exposed chromophoric residues can be obtained by comparing the $\Delta\epsilon_{\lambda,\max}$ of the protein with the $\Delta\epsilon_{\lambda,\max}$ of its model compound mixture in the same solvent.

The formula for such a calculation is:

$$R_M = \frac{(\Delta\epsilon_{\lambda,\max}/\epsilon_{\lambda,\max})_{\text{protein}}}{(\Delta\epsilon_{\lambda,\max}/\epsilon_{\lambda,\max})_{\text{model cpd}}} \quad (2)$$

Estimates of the fraction of exposed tryptophyl residues (given in parentheses in Table I) are based on model compound mixtures in water. It is worth noting that when appropriate corrections are made for the effects of urea, estimates based on the disulfide-cleaved protein and on model compound mixtures both yield essentially the same information. It should also be noted that, while in lysozyme only about one third of the tryptophyl residues seem to be buried, in α -chymotrypsinogen on the order of 60% of the tryptophyl residues appear to be inaccessible to solvent.

The use of the difference spectral constant, $\Delta\epsilon_{\lambda,\max}/\epsilon_{\lambda,\max}$, for the representation of solvent perturbation data has the advantage over the commonly employed molar quantities in that neither the exact tyrosine and tryptophan contents nor other macromolecular constants, such as the molecular weight, the molar absorptivity, or the exact protein concentration of solutions employed, need to be known. The only auxiliary information required in addition to the measured absorbancy difference of a given solution due to a particular perturbant is the absolute value of the absorbancy, since $\Delta A_{\lambda,\max}/A_{\lambda,\max} = \Delta\epsilon_{\lambda,\max}/\epsilon_{\lambda,\max}$.

¹⁷ L. Weil, T. S. Seibles, and T. T. Herskovits, *Arch. Biochem. Biophys.* **111**, 308 (1965).

For proteins in which both tyrosine and tryptophan contribute materially to the difference spectrum, that is, where the molar ratio of tyrosine to tryptophan is in the neighborhood of 4:1, in principle an estimate of the number or fraction of tyrosines and tryptophans exposed can be obtained by solving two simultaneous equations of the sort,

$$\Delta\epsilon_{286-288}(\text{protein}) = a \times \Delta\epsilon_{286-288}(\text{tyr}) + b \times \Delta\epsilon_{286-288}(\text{trp}) \quad (3)$$

$$\Delta\epsilon_{292-294}(\text{protein}) = a \times \Delta\epsilon_{292-294}(\text{tyr}) + b \times \Delta\epsilon_{292-294}(\text{trp}) \quad (4)$$

where coefficients a and b represent the number of tyrosines and tryptophans exposed; the $\Delta\epsilon_{\lambda}$ values refer to the experimentally determined molar absorptivity differences of the protein, free tyrosine (tyr), and tryptophan (trp), respectively, in the wavelength region designated by the subscripts.

There are a number of difficulties with this latter approach that should be emphasized. In native proteins the two difference spectral maxima at 286–288 $m\mu$ and 292–294 $m\mu$ are often rather poorly resolved. This may be due to the fact that the environments in which the various exposed or partly exposed groups are located are not the same, or possibly that some of the groups participate in short-range interactions with neighboring amino acid side chains. In addition, incorporation of the chromophoric residues into the polypeptide backbone is known to produce a slight shift in the difference spectrum toward longer wavelengths,^{13,18,19} so that the tyrosine and tryptophan peaks in proteins need not coincide exactly with the peaks of their model compound analogs (see Fig. 3 concerning this point). For these reasons estimates of the number or fraction of exposed chromophores based on Eqs. (3) and (4) are at best approximate. Perhaps a more satisfactory approach to this problem, suggested by Donovan,¹⁹ consists of fitting the difference spectral profile of the protein in question with appropriate combinations of tyrosine and tryptophan curves based on model experiments. Such results with native and acid-denatured aldolase, obtained with 20% ethylene glycol as perturbant, are reproduced in Fig. 3. In the native form at pH 6, the best fit has been obtained with 14 moles of tyrosine and 1 mole of tryptophan (lower curve represented by dashed line) out of a total of 42 moles of tyrosine and 10 moles of tryptophan per mole of protein, suggesting that most of the tryptophans and about two thirds of the tyrosines are buried in the interior folds of the native protein. It is interesting that the difference spectrum of the acid-denatured protein at pH 2 is best fitted with 42

¹⁸ G. H. Beaven and E. R. Holiday, *Advan. Protein Chem.* **7**, 319 (1952).

¹⁹ J. W. Donovan, *Biochemistry* **3**, 67 (1964).

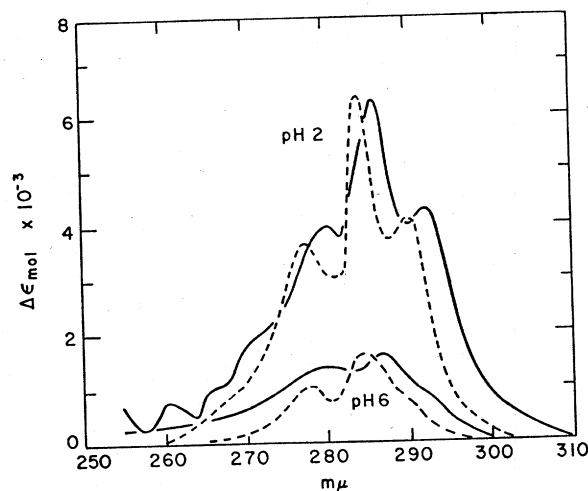


FIG. 3. Solvent perturbation difference spectra of aldolase produced by 20% ethylene glycol. The difference spectra of the protein (*solid lines*) are compared with the theoretical curves of model compounds (*dashed lines*) calculated for 10 moles of tryptophan plus 42 moles of tyrosine (at pH 2) and 1 mole of tryptophan plus 14 moles of tyrosine (at pH 6). (The difference spectra of the individual amino acids on the basis of which the theoretical curves were constructed are not shown here.) The aldolase concentration was $1.6 \times 10^{-5} M$ [from J. W. Donovan, *Biochemistry* 3, 67 (1964)].

moles of tyrosine and 10 moles of tryptophan (dashed upper curve), suggesting that at this pH the protein is fully unfolded, with all the chromophoric residues coming freely in contact with the solvent.

Auxiliary Measurements. One of the main assumptions of the solvent perturbation technique is that the perturbant does not alter the conformation of the particular protein under investigation. Herskovits and Laskowski⁷ suggested that the effect of the perturbing solvent on some of the physical properties of the proteins in question should be investigated. The macromolecular parameters that are sensitive to small conformational changes and can be fruitfully employed for this purpose are the intrinsic or reduced viscosity, the sedimentation coefficient, the optical rotatory dispersion, and the acid difference spectra.^{7, 20}

Sucrose, ethylene glycol, glycerol, and dimethylsulfoxide seem to be the most reliable perturbants for solvent perturbation studies.^{7, 15} The first three of these substances are known to stabilize the native conforma-

tion of proteins.²¹ Hamaguchi and co-workers^{22,23} have found that none of these perturbants causes significant changes in specific viscosity of lysozyme at the 20 volume percent level. In addition, at this concentration most of these perturbants have little or no effect on the rotatory dispersion parameters and, in cases where such studies have been made, on the acid difference spectra of such proteins as the serum albumins,⁷ lysozyme, β -lactoglobulin,²⁴ and α -lactalbumin.²⁰

Application. In addition to the studies of lysozyme and α -chymotrypsinogen, the solvent perturbation technique has been applied to the study of a number of other enzymes and proteins.^{6,7,14-17,20} Studies on the serum albumins⁷ and ovomucoid¹⁴ have suggested that the perturbation spectra produced by additives of different molecular dimensions may also be used to explore the topology and geometry of proteins composed of subunits and of proteins possessing crevices and folds that may be selectively permeable to solvents. In the case of serum albumin,⁷ for example, it was observed that perturbants with mean molecular diameters equal to or greater than 5.2 Å (glycerol, sucrose, and polyethylene glycol) (Table II)

TABLE II
MEAN DIAMETERS OF ADDITIVES (PERTURBANTS) EMPLOYED
IN SOLVENT PERTURBATION SPECTRAL WORK

Additive	Mean diameter ^a (Å)
Heavy water	2.2
Dimethylsulfoxide	4.0
Ethylene glycol	4.3
Glycerol	5.2
Erythritol	5.8
Arabitol	6.4
Glucose	7.2
Mannitol	7.4
Polyethylene glycol	9.2
Sucrose	9.4

^a Calculated by means of the Stokes-Einstein relation; diameter = $2kT/6\pi\eta_0D$. The diffusion constant D is taken from the literature. The dimensions of dimethylsulfoxide $[(CH_3)_2S=O]$ are taken to be the same as those of acetone $[(CH_3)_2C=O]$. [From T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.* **237**, 2481 (1962); and M. S. Schormuller, Ph. D. Thesis, Purdue University, 1965.]

²¹ R. B. Simpson and W. Kauzmann, *J. Am. Chem. Soc.* **75**, 5139 (1953).

²² K. Hamaguchi, K. Hayashi, T. Imoto, and M. Funatsu, *J. Biochem. (Tokyo)* **55**, 24 (1964).

²³ K. Hamaguchi, *J. Biochem. (Tokyo)* **56**, 441 (1964).

²⁴ C. Tanford, P. K. De, and V. G. Taggart, *J. Am. Chem. Soc.* **82**, 6028 (1960).

are excluded from the postulated interfaces between conformational subunits of this protein in the neutral pH region. When these conformational subunits separate at the well-known acid transition around pH 4, another 20% of the tyrosines (3-5 residues in addition to the 6-8 exposed residues) become accessible to these bulky perturbants (see the polyethylene glycol curve of Fig. 4). In contrast, the interfaces between conformational

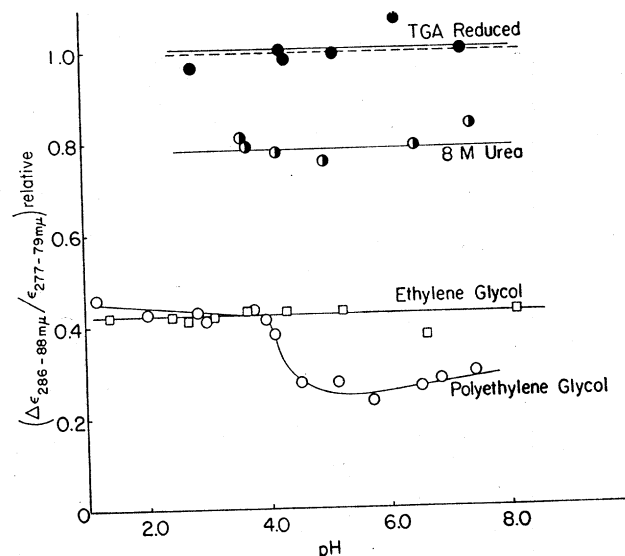


FIG. 4. The effect of perturbant size on the solvent perturbation difference spectra of bovine serum albumin (BSA) at 286-288 $m\mu$ as a function of pH. Data are expressed relative to the $\Delta\epsilon_{\lambda, \max}/\epsilon_{\lambda, \max}$ values obtained with the disulfide-cleaved protein in 8 M urea. Perturbants: \square , 20% ethylene glycol, $\Gamma/2 = 0.25$; ---, 20% ethylene glycol, thioglycolic acid-reduced BSA in 8 M urea; \circ , 20% polyethylene glycol, $\Gamma/2 = 0.25$; \bullet , 20% polyethylene glycol, 8 M urea, $\Gamma/2 = 0.25$; \bullet , 20% polyethylene glycol, thioglycolic acid-reduced BSA in 8 M urea, $\Gamma/2 = 0.2$ [from T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.* **237**, 2481 (1962)].

subunits are permeable to perturbants having mean molecular diameters equal to or less than 4.3 Å (i.e., ethylene glycol and dimethylsulfoxide?) throughout both the neutral and acid pH region. As a result these smaller perturbants may "see" the partially accessible tyrosyl residues, which are located at the conformational subunit interfaces, throughout the whole pH region. This is strongly suggested by the fact that the difference spectral constants obtained with these perturbants are unaffected by change in pH and are relatively high when compared to the constants due to the bulkier perturbants in the neutral pH region (e.g., compare the ethylene glycol curve to the polyethylene glycol curve of Fig. 4).

These observations are in accord with the model for serum albumin proposed by Foster.²⁵ In this model, the peptide chain of the protein is folded into four flat conformational subunits stacked one on top of the other, with three interfaces between them. The reader is referred to the original literature on this subject, including the findings on ovomucoid, and the recent studies of Kronman and Holmes on α -lactalbumin.^{7, 14, 20}

Hamaguchi and co-workers^{22, 23, 26-29} have studied the difference spectral properties of lysozyme that are produced by a variety of salts and organic solvents. They have also examined the changes in optical rotatory properties and reduced viscosity of this enzyme as a function of the concentration of these reagents and salts. Figure 5 represents the changes found in these parameters as a function of concentration of acidic methanol. The method of analysis of their difference spectral data was suggested by the studies of Bigelow and Geschwind³⁰ and Bigelow³¹ on the urea and lithium bromide denaturation of ribonuclease. This so-called slope method consists in comparing the initial slope of the protein curve, $\Delta\epsilon_{\lambda, \max}$ versus denaturant or perturbant concentration, with the slope of a similar curve obtained with model compounds (tryptophan or leucyl-tryptophan was employed in the case of lysozyme). The apparent number of chromophoric residues exposed to the perturbing influence of the solvent simply is equal to the ratio of the molar absorptivity difference of the protein to that of the model, per mole or unit volume of denaturant employed. Their conclusion is that four of the six tryptophyl residues in lysozyme are exposed and accessible to solvent; the remaining two groups are buried in the interior of the protein. This conclusion is essentially the same as that of Williams *et al.*,¹⁵ based on solvent perturbation studies, which was discussed earlier in the text (see also Fig. 2). It should be noted, however, that this conclusion is not unexpected, since the two difference spectral methods are based on the same physical phenomenon, and therefore should not be taken as proof of the number or fraction of exposed chromophoric residues, which might be the case in application of two truly independent methods.

It should also be emphasized that the difference spectral technique measures only the fraction of exposed groups; therefore the interpretation

²⁵ J. F. Foster, in "The Plasma Proteins" (F. W. Putnam, ed.), Vol. 1, p. 179. Academic Press, New York, 1960.

²⁶ K. Hamaguchi and A. Kurono, *J. Biochem. (Tokyo)* **54**, 111 (1963).

²⁷ K. Hamaguchi, A. Kurono, and S. Goto, *J. Biochem. (Tokyo)* **54**, 259 (1963).

²⁸ K. Hamaguchi and A. Kurono, *J. Biochem. (Tokyo)* **54**, 497 (1963).

²⁹ A. Kurono and K. Hamaguchi, *J. Biochem. (Tokyo)* **56**, 432 (1964).

³⁰ C. C. Bigelow and I. I. Geschwind, *Compt. Rend. Trav. Lab. Carlsberg* **31**, 283 (1960).

³¹ C. C. Bigelow, *Compt. Rend. Trav. Lab. Carlsberg* **31**, 305 (1960).

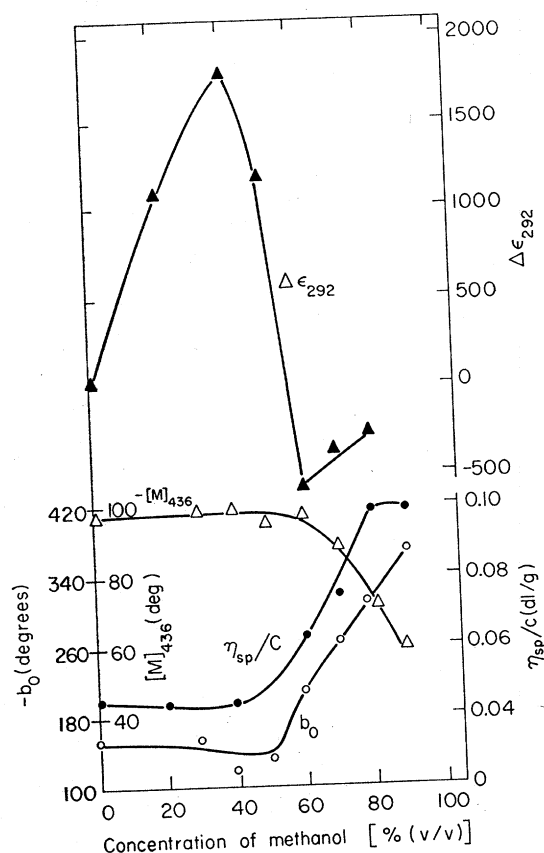


FIG. 5. Variation of the molar absorptivity difference at 292 m μ , $\Delta\epsilon_{292}$, the reduced viscosity, η_{sp}/C , the mean residue rotation at 436 m μ , $[M]_{436}$, and the Moffitt rotatory dispersion parameter, b_0 , of lysozyme as a function of methanol concentration. The aqueous alcohol solutions contained 0.005 M HCl [from A. Kurono and K. Hamaguchi, *J. Biochem. (Tokyo)* **56**, 432 (1964)].

of solvent perturbation data, in terms of fully exposed and buried groups, is at best a shrewd estimate or an approximate description of the true situation. From X-ray crystallographic studies on horse hemoglobin³² and sperm whale myoglobin,³³ it may be presumed that some of the tyrosine and tryptophan residues in these proteins are only partly buried. Such partly buried residues would be expected to contribute to the solvent per-

³² M. F. Perutz, M. G. Rossmann, A. F. Cullis, H. Muirhead, G. Will, and A. C. T. North, *Nature* **185**, 416 (1960).

³³ J. C. Kendrew, R. E. Dickerson, B. E. Strandberg, R. G. Hart, D. R. Davies, D. C. Phillips, and V. C. Shore, *Nature* **185**, 422 (1960).

turbation difference spectra of proteins. In fact some evidence, based on oxidation of tryptophyl residues in lysozyme by *N*-bromosuccinimide, has been presented³⁴ that lends support to the idea that in lysozyme the difference spectral parameters reflect the presence of several fully exposed residues and some partly buried residues, rather than four fully exposed and two completely buried tryptophyl residues. Furthermore, these observations are in accord with the spatial disposition of the chromophoric groups revealed by recent X-ray diffraction studies on lysozyme in the crystalline state.³⁵ The application of the tryptophyl oxidation method³⁴ together with solvent perturbation difference spectroscopy to α -chymotrypsinogen, on the other hand, has suggested that in this protein three tryptophans are exposed and four are buried.¹⁵

The solvent perturbation technique has also been extended to the study of proteins and polypeptides in helix-promoting and random coil-promoting organic solvents.³⁶ These studies have shown that in solvents such as methanol, 2-chloroethanol, and in some cases ethylene glycol, just as in 8 *M* urea (a random coil-promoting solvent), a large fraction of the normally buried chromophoric residues are exposed to solvent. Since the solubility of chromophoric amino acids and model compounds corresponding to these amino acids is greatly enhanced in organic solvents,^{36,37} it has been suggested that the exposure of these amino acids in proteins is due to the destruction of hydrophobic regions in which some of their side chains are buried. In this connection, information about the relative importance of hydrophobic interactions can be gained from the effect of ethylene glycol on such hydrophobic regions in proteins. It is generally believed³⁸⁻⁴⁰ that this solvent has little or no effect on hydrogen bond-stabilized regions in proteins; with other stabilizing factors kept more or less constant, the extent of unfolding or exposure of chromophoric side chains by this solvent can give some measure of the importance of hydrophobic interactions in globular proteins.

Wang and Laskowski⁴¹ have recently found that the solvent perturba-

³⁴ E. J. Williams and M. Laskowski, Jr., *J. Biol. Chem.* **240**, 3580 (1965).

³⁵ C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature* **206**, 757 (1965).

³⁶ C. Tanford, *J. Am. Chem. Soc.* **84**, 4240 (1962).

³⁷ D. B. Wetlaufer, S. K. Malik, L. Stoller, and R. L. Coffin, *J. Am. Chem. Soc.* **86**, 508 (1964).

³⁸ S. J. Singer, *Advan. Protein Chem.* **17**, 1 (1962).

³⁹ C. Tanford, C. E. Buckley, III, P. K. De, and E. P. Lively, *J. Biol. Chem.* **237**, 1168 (1962).

⁴⁰ J. Brahms and C. M. Kay, *J. Biol. Chem.* **237**, 3449 (1962).

⁴¹ C. C. Wang, Ph.D. Thesis, Purdue University, 1963; M. Laskowski, Jr., *Federation Proc.* **25**, 20 (1966).

tion technique is also well suited to study of the location of chromophoric groups artificially introduced into proteins. The location of 1-dimethylamino-5-naphthalenesulfonyl (DNS) chromophores, coupled to lysozyme, ovomucoid, and serum albumin, was studied by these workers. It was found, for example, that the interior of serum albumin can accommodate the chromophore more readily than can the interior of lysozyme or ovomucoid. Interestingly, DNS coupled to serum albumin has an abnormally low pK value.⁴² In sharp contrast to the findings with serum albumin, in lysozyme and ovomucoid the pK values are almost normal and the DNS chromophores are found to be very nearly fully exposed.

Experiments such as these should lead to useful information in other studies on dye-protein or hapten-protein conjugates or complexes. In addition, it should be possible to study complex formation between antigens and antibodies, and between enzymes and substrates. In connection with the latter, mention should be made of the recent study by Hayashi and co-workers⁴³ on lysozyme-glycol chitin complex formation by the slope method of solvent perturbation discussed above. These workers have found that in the process of enzyme-substrate complex formation some of the exposed or partly exposed tryptophyl residues become buried. Other evidence has been presented in the literature^{44, 45} that also implicates some of the tryptophyl residues in the activity of this protein.

Acid and Alkali-Induced Difference Spectra

Unlike solvent-induced shifts and difference spectra, the origin of which can be attributed to a single phenomenon (i.e., fully and partly exposed aromatic side chains in proteins), acid- and alkali-induced difference spectra in proteins owe their origin to a number of interrelated physical phenomena. The difference spectra of proteins due to pH changes have been the subject of a number of recent articles and reviews.^{13, 46-49} The pH-induced difference spectra in the 250-300 $m\mu$ region have been ascribed to the following causes:

(1) Changes in distribution of positive and negative charges in the vicinity of the chromophores (i.e., vicinal charge effects).

⁴² I. M. Klotz and H. A. Fiess, *Biochim. Biophys. Acta* **38**, 57 (1960).

⁴³ K. Hayashi, T. Imoto, and M. Funatsu, *J. Biochem. (Tokyo)* **55**, 516 (1964).

⁴⁴ L. Weil, A. R. Buchert, and J. Maher, *Arch. Biochem. Biophys.* **40**, 245 (1952).

⁴⁵ G. J. S. Rao and L. K. Ramachandran, *Biochim. Biophys. Acta* **59**, 507 (1962).

⁴⁶ D. B. Wetlaufer, J. T. Edsall, and B. R. Hollingworth, *J. Biol. Chem.* **233**, 1431 (1958).

⁴⁷ S. Yanari and F. A. Bovey, *J. Biol. Chem.* **235**, 2818 (1960).

⁴⁸ S. J. Leach and H. A. Scheraga, *J. Biol. Chem.* **235**, 2827 (1960).

⁴⁹ H. A. Scheraga, "Protein Structure." Academic Press, New York, 1961.

(2) Breaking and formation of side-chain hydrogen bonds involving tyrosyl residues and possibly tryptophyl residues.

(3) Changes in polarity and polarizability of the chromophore environment due to structural alteration of the protein.

(4) Ionization of tyrosyl residues at high pH.

Acid Difference Spectra. The usual changes encountered in the difference spectral parameters of proteins upon acidification are of the order of -80 to -500 absorbancy units per mole of chromophore.⁴⁷ The acid titration of ribonuclease and serum albumin, for example, is accompanied by a change in tyrosyl absorptivity at $287\text{ m}\mu$ of approximately -150 to -370 . In α -lactalbumin and lysozyme—two proteins with difference spectra dominated by tryptophan—the molar absorptivity difference is of the same order, that is, about -400 and -80 to -150 units, respectively. Hydrodynamic studies indicate that at least the first three of these proteins undergo limited structural alterations in acid,⁴⁹⁻⁵² while lysozyme, on the other hand, seems to be unaffected by change in pH.¹⁰ Moreover, while there is an increase in the fraction of exposed tyrosyls in serum albumin⁷ and ribonuclease,⁶ as suggested by solvent perturbation experiments, no corresponding change in the fraction of exposed tryptophyl residues is found in the case of α -lactalbumin²⁰ or lysozyme.¹⁵

It thus appears that limited changes in the absorptivity difference of proteins, that is, changes of the order of -100 to -150 units per mole of chromophore, cannot be interpreted in terms of conformational changes alone. Nor can such changes due to acidification be unequivocally assigned to hydrogen bond breakage or vicinal charge effects. At present these effects cannot be separated and assigned on the basis of known differences in the sign, the magnitude, or the shape or position of the difference spectral maxima and minima.^{30, 46} Acid difference spectra, denaturation difference spectra, and solvent perturbation difference spectra are all remarkably similar as far as the shape and position of the peaks are concerned; differences usually occur in the sign and magnitude of the shifts or absorbancy differences.⁵³

The complete unfolding of proteins is usually accompanied by larger changes in the absorbancy. For example, with ribonuclease the unfolding

⁵⁰ J. T. Yang and J. F. Foster, *J. Am. Chem. Soc.* **76**, 1588 (1954).

⁵¹ C. Tanford, J. G. Buzzell, D. G. Rands, and S. A. Swanson, *J. Am. Chem. Soc.* **77**, 6421 (1955).

⁵² M. J. Kronman, L. Cerankowski, and L. G. Holmes, *Biochemistry* **4**, 518 (1965).

⁵³ With the 20% perturbants commonly employed (Table I) the $\Delta\epsilon_{\lambda, \text{max}}$ values per mole of chromophore usually vary from about $+20$ to $+100$ (due to 20% sucrose) to about $+60$ to $+350$ (due to 20% dimethylsulfoxide).

process in strong urea solutions (Fig. 6A) is accompanied by a net change³⁰ at 287 $m\mu$ of -450 absorbancy units per mole of tyrosine. The acid denaturation of aldolase is accompanied by a larger change of about -1600 absorbancy units per mole of tryptophan (at 293 $m\mu$).¹⁹ As would be expected, this change in the absorbancy of aldolase is accompanied by complete exposure of the chromophoric residues (Fig. 3).

With the above examples from the literature in mind, it is recommended that acid difference spectral measurements always be accompanied by parallel hydrodynamic measurements (viscosity, ultracentrifugal analysis) and solvent perturbation experiments. Information on the effect of urea or other unfolding agents on the absorbance should also facilitate the interpretation of such data. Fortified with such information, a reasonable interpretation of acid difference spectra may be made.

Spectrophotometric Titration at High pH. The main spectral changes observed with tyrosine-containing proteins in the alkaline pH region are due to the ionization of tyrosyl residues. The ionization of tyrosine is accompanied by both an intensification of the spectrum and a shift of the maximum from 275 to 293 $m\mu$.

The ionization of tyrosine in proteins is most conveniently studied by difference spectral methods, since the change in molar absorptivity at 293–295 $m\mu$ on full ionization is in the neighborhood of 2300 units.^{13, 18} Usually the pH of test solutions is varied and spectra are determined against a reference solution of identical concentration at neutral pH. Free tyrosine ionizes with an apparent pK of 9.7. In proteins, some of the tyrosines often ionize at much higher pK values. The abnormally high pK values associated with these residues have been attributed to the fact that the residues are masked or buried and thus shielded from reagent, or perhaps are hydrogen-bonded to other side chains of the protein. Figure 6B shows the spectrophotometric titration of ribonuclease and two ribonuclease derivatives, ribonuclease partially digested with pepsin and performic acid-oxidized ribonuclease. As shown in this figure, the native enzyme (data represented by open circles) titrates in two waves: half of the ionization curve (up to $\Delta\epsilon_{295} = 8000$) has a midpoint at pH 10, while the remaining steeper part of the curve (representing the ionization of three masked tyrosyls) has a midpoint above pH 12. This upper portion of the titration curve is also time-dependent and partly irreversible, suggesting that the protein has to unfold before these masked residues can be ionized.^{54, 55} In the partially digested protein (data

⁵⁴ D. Shugar, *Biochem. J.* **52**, 142 (1952).

⁵⁵ C. Tanford, J. D. Hauenstein, and D. G. Rands, *J. Am. Chem. Soc.* **77**, 6409 (1955).

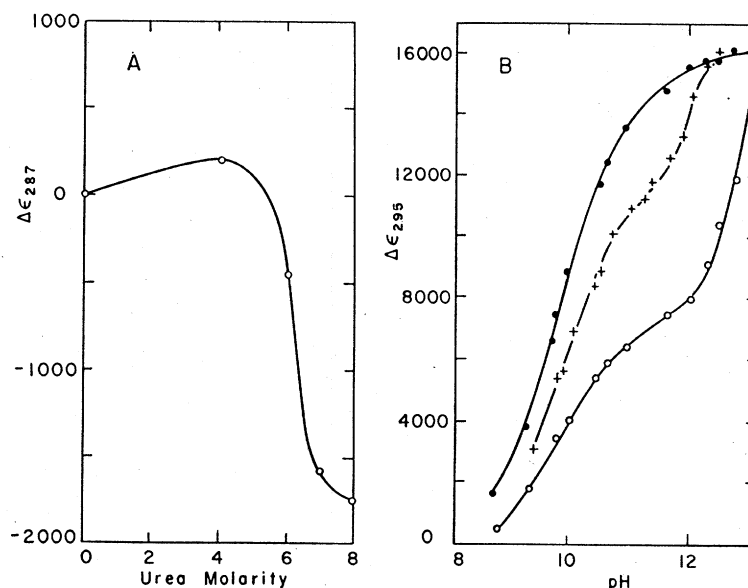


FIG. 6. The effect of urea and alkali on the difference spectral parameters of ribonuclease: (A) effect of urea concentration on the molar absorptivity difference of ribonuclease at 287 $m\mu$ [from C. C. Bigelow and I. I. Geschwind, *Compt. Rend. Trav. Lab. Carlsberg* 31, 283 (1960)]; (B) spectrophotometric titration of ribonuclease and two ribonuclease derivatives at high pH. O, ribonuclease; +, ribonuclease partially digested with pepsin; ●, performic acid-oxidized ribonuclease [from C. C. Bigelow, *Compt. Rend. Trav. Lab. Carlsberg* 31, 305 (1960)].

represented by crosses) about five of the six tyrosyls seem to ionize normally, while in oxidized ribonuclease all six of the tyrosyls appear to ionize normally with an apparent pK of about 10 (i.e., the pH value at the mid point of the titration curve). The suggestion that the three residues that titrate abnormally in ribonuclease are in fact buried has been borne out by recent solvent perturbation studies⁶ and by iodination studies.^{56, 57}

In contrast to ribonuclease, in which the three abnormal tyrosyls ionize only after irreversible structural changes in the protein have taken place, in bovine serum albumin all the tyrosyls ionize reversibly, but the pK is somewhat higher than expected ($pK_{\text{intrinsic}} = 10.35^{58, 59}$ as compared to 9.5–10 obtained with other normally ionizing groups in proteins and with free tyrosine⁵⁵) and the heat of ionization is also abnormally high.^{58, 59} The fact that about 70% of the tyrosyls are buried in bovine serum albumin⁷ suggests that spectrophotometric titration data must

⁵⁶ C. Y. Cha and H. A. Scheraga, *J. Biol. Chem.* 238, 2965 (1963).

⁵⁷ L. Gruen Donovan, *Biochim. Biophys. Acta* 78, 474 (1963).

⁵⁸ C. Tanford and G. L. Roberts, Jr., *J. Am. Chem. Soc.* 74, 2509 (1952).

⁵⁹ C. Tanford, *Advan. Protein Chem.* 17, 69 (1962).

also be interpreted with caution and with due consideration of the literature of the subject.^{18, 54, 55, 58, 59}

Estimates of the apparent heats and entropies of ionization can be obtained from the temperature dependence of the reversible portion of spectrophotometric titration curves. (The irreversible portion of these curves is not amenable to thermodynamic interpretation.) These parameters can be calculated from slopes of plots of apparent pK versus $1/T$ by use of the relationships

$$\Delta H = 2.303R[\Delta pK/\Delta(1/T)] \quad (5)$$

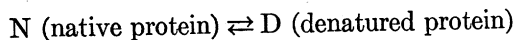
$$\Delta S = \Delta H/T - 2.303RpK \quad (6)$$

where ΔH and ΔS are the apparent heat and entropy of ionization, R is the gas constant, T is the absolute temperature, and $\Delta pK/\Delta(1/T)$ is the slope. Where hydrogen ion titration curves are available (see this volume [84]) these calculations should be based on intrinsic rather than apparent pK values. This correction takes into account the effects of electrostatic interactions, due to the presence of other charges on the protein. The correction is usually of the order of -0.1 to -0.4 pK unit. Normal heats and entropies of ionization are in the neighborhood of 6 kcal and -26 entropy units per mole of tyrosine, while abnormal values can be as high as 11.5 kcal and as low as -9 entropy units, respectively.

Temperature Difference Spectra

The study of the thermal denaturation of proteins by difference spectral methods is of fairly recent origin.⁶⁰⁻⁶³ As in the case of urea or acid denaturation, the destruction of the secondary and tertiary structure of proteins at elevated temperatures is accompanied by similar spectral changes or shifts (Fig. 7). Studies on such proteins as ribonuclease,^{61, 64} lysozyme,⁶⁰ and α -chymotrypsinogen^{62, 63} indicate that these spectral or difference spectral changes (so-called denaturation blue shifts) as a rule will be accompanied by parallel changes in other optical or hydrodynamic properties (optical rotation, specific viscosity, sedimentation coefficients, etc.). When such parallel or auxiliary studies indicate a single denaturation process, difference spectroscopy offers a convenient method for obtaining the thermodynamic parameters of the process.

If we assume that the denaturation process is a reversible single step reaction,



⁶⁰ J. G. Foss, *Biochim. Biophys. Acta* **47**, 569 (1961).

⁶¹ J. Hermans, Jr. and H. A. Scheraga, *J. Am. Chem. Soc.* **83**, 3283 (1961).

⁶² J. F. Brandts and R. Lumry, *J. Phys. Chem.* **67**, 1484 (1963).

⁶³ J. F. Brandts, *J. Am. Chem. Soc.* **86**, 4291, 4302 (1964).

⁶⁴ D. N. Holcomb and K. E. Van Holde, *J. Phys. Chem.* **66**, 1999 (1962).

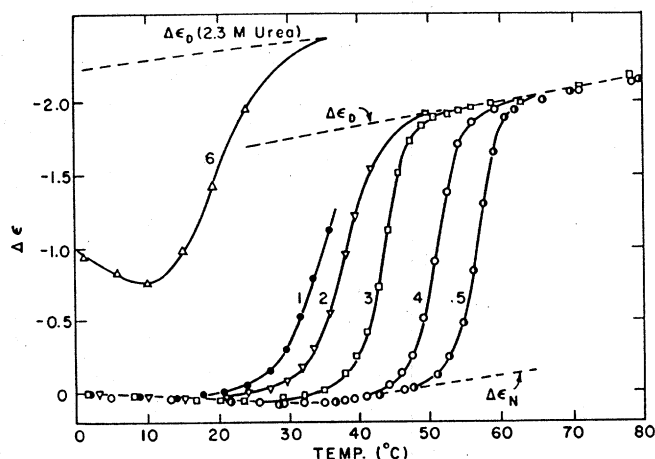


Fig. 7. The temperature dependence of the absorbivity ($\epsilon_{\text{lem}}^{1\%}$) of chymotrypsinogen at $293 \text{ m}\mu$: curve 1, pH 1.11; curve 2, pH 1.71; curve 3, pH 2.07; curve 4, pH 2.56; curve 5, pH 3.00; curve 6, 2.3 *M* urea, pH 1.55; all solutions contained only HCl (no added salt) [from J. F. Brandts, *J. Am. Chem. Soc.* **86**, 4291 (1964)].

then, from the equilibrium constant K for this process, the various thermodynamic parameters of the denaturation can be estimated. In terms of spectrophotometric denaturation data (shown in Fig. 7), the equilibrium constant can be expressed as:

$$K = f/(1 - f) = (\epsilon - \epsilon_N)/(\epsilon_D - \epsilon) \quad (7)$$

Here f represents the fraction of molecules in the denatured state and the subscripts N and D refer to the absorbivity of the protein in the native and denatured states, respectively; ϵ is the absorbivity of the proteins in the transition region. As shown in Fig. 7, these reference values of ϵ can vary slightly with temperature, but it should be clear that this poses no problem to evaluation of the data. Since the difference spectral technique yields only absorbancy differences, the calculation of the equilibrium constant will ordinarily be made from the equivalent relation,⁶³

$$K = (\Delta\epsilon - \Delta\epsilon_N)/(\Delta\epsilon_D - \Delta\epsilon) \quad (8)$$

where all the absorbivities have been reduced by the same amount by subtracting the absorbivity of an arbitrary reference solution.

The standard free energy of denaturation can be calculated by use of the relation

$$\Delta F^\circ = -RT \ln K \quad (9)$$

while the standard-state heat or enthalpy, ΔH° , and entropy, ΔS° , changes can be estimated with the usual relations:

$$\Delta H^\circ = -R\Delta \ln K / \Delta(1/T) \quad (10)$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta F^\circ)/T = \Delta H^\circ/T + R \ln K \quad (11)$$

ΔH° can be evaluated from the slope of a van't Hoff plot (i.e., a plot of $R \ln K$ versus $1/T$), while ΔS° can be conveniently evaluated from the determined ΔH° at a temperature, $T_{1/2}$, corresponding to the midpoint of the denaturation transition curve (i.e., $R \ln K = 0$ at $T_{1/2}$).

Unfortunately the van't Hoff plots usually show some curvature at high temperatures, apparently as a consequence of the differences in heat capacity of native and denatured proteins.⁶³ For these reasons the heat and entropy changes derived will give only a very approximate idea of the relative stability of a given protein, that is, the amount of free energy that must be overcome to denature the protein at ordinary temperatures.

Brandts⁶³ has recently shown that it is possible to obtain meaningful thermodynamic parameters over a fairly wide range of experimental conditions by indirect methods. His method consists of fitting the curve of experimentally obtained ΔF° versus absolute temperature, T , by a power series involving T , and evaluation of the free energy contributions arising from hydrogen bonding, hydrophobic interactions, conformational entropy, electrostatic interaction, and abnormally ionizing side chains from the coefficients of this series. Details of his method, in terms of these stabilizing and destabilizing factors, will not be presented here; the reader is referred to the original articles.⁶³ The generality of his conclusions, based on the denaturation of chymotrypsinogen, suggests that, in the case of many globular proteins at ordinary temperatures, hydrogen bonding and hydrophobic bonding should contribute about equally to stabilization of the native conformation. It is significant that these two stabilizing effects are very nearly overcome by the large destabilization free energy which arises from the increase in conformation entropy. In the case of native α -chymotrypsinogen, hydrogen bonding seems to contribute about 120 kcal of stabilization free energy (largely independent of temperature). The contribution from hydrophobic interactions is about the same at room temperature. (A moderate increase in these interactions is observed at higher temperatures, leveling off at about 140 kcal at 70° and then slightly decreasing.) These two stabilizing effects are very nearly canceled by the large destabilizing conformational entropy term, which varies linearly from about -210 kcal at 0° to -270 kcal at 70°. Thus Brandts concludes that, although there may be well over 250 kcal of stabilizing free energy, the net free energy of this protein will seldom exceed 10 kcal. Theoretical models for the examination of the temperature

dependence of the denaturation process of proteins, in terms of abnormally behaving titratable groups and other factors, have also been presented by Hermans and Scheraga⁶¹ and by Sophianopoulos and Weiss.⁶⁵

Other Applications of Difference Spectroscopy

In the past few years difference spectral techniques have found fairly extensive application in denaturation studies of proteins by means of a variety of denaturing agents and salts.^{22, 23 26-31} The kinetics of denaturation^{66, 67} and renaturation (e.g., ribonuclease⁶⁸) have been studied by this technique. The proteolysis of enzymes has been determined by this technique. Richards and Logue⁶⁸ have recently studied the spectral changes and kinetics of complex formation in subtilisin-treated ribonuclease, between the so-called S-protein (consisting of residues 21-124 of the parent protein) and the S-peptide of ribonuclease (residues 1-20 of the parent protein).

Spectral and difference spectral measurements have been extended into the region below 250 m μ . Glaser and Smith^{69, 70} have reported the presence of a large peak in the acid difference spectra of a number of proteins, centering around 235 m μ . They have suggested that this peak may be due to changes in absorption of the polypeptide backbone of proteins. In the case of serum albumin⁷¹ and α -lactalbumin,⁵² however, changes in this spectral band have been found to parallel changes in the peaks at 287 m μ (tyrosine) and 293 m μ (tryptophan). This is, of course, not surprising since the amino acids tyrosine, tryptophan, phenylalanine, histidine, methionine, and cystine are known to contribute to the protein spectrum in this wavelength region.¹³ Donovan^{19, 72} has recently extended the analysis in this spectral region by means of difference spectroscopy for the titration of sulfhydryl and imidazole groups in proteins.

The difference spectral techniques discussed in this chapter should also find application to problems not so far discussed. In principle it should be possible to extend the method to problems dealing with the mechanism and kinetics of a variety of protein-protein, protein-ligand, and protein-small molecule interactions, provided, of course, such inter-

⁶⁵ A. J. Sophianopoulos and B. J. Weiss, *Biochemistry* **3**, 1920 (1964).

⁶⁶ C. A. Nelson and J. P. Hummel, *J. Biol. Chem.* **237**, 1567 (1962).

⁶⁷ A. N. Glaser and H. A. McKenzie, *Biochim. Biophys. Acta* **65**, 526 (1962).

⁶⁸ F. M. Richards and A. D. Logue, *J. Biol. Chem.* **237**, 3693 (1962).

⁶⁹ A. N. Glaser and E. L. Smith, *J. Biol. Chem.* **235**, PC43 (1960).

⁷⁰ A. N. Glaser and E. L. Smith, *J. Biol. Chem.* **236**, 2942 (1961).

⁷¹ Studies of D. Eisenberg. Cited by J. T. Edsall, in "Aspects of Protein Structure" (G. N. Ramachandran, ed.), p. 179. Academic Press, New York, 1963.

⁷² J. W. Donovan, *Biochemistry* **4**, 823 (1965).

actions are accompanied by spectral changes. The double-cell method of solvent compensation (described below under "Experimental Procedures") should be of considerable utility in such studies.

Experimental Procedures

Instrumentation and Instrumental Methods. Many of the more recent difference spectral studies have been carried out with the aid of recording double-beam instruments, such as the Cary Model 14 or 15 spectrophotometers. The use of double-compartment cells designed to subtract the solvent contributions to the difference spectra,⁷ especially in the case of studies with strongly absorbing additives, has made these measurements a relatively routine matter. However, because the difference spectra of proteins and related model substances may often constitute only a few percent of their direct spectra, the care with which instrumental measurements and volumetric manipulations must be carried out in order to obtain meaningful results cannot be overemphasized.

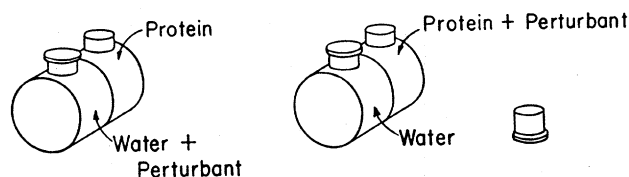


FIG. 8. Tandem double-compartment cells employed in solvent perturbation spectral work. The cells are manufactured (Pyrocell Mfg. Co., Westwood, New Jersey) to specification, with 1.0-cm or 4.50-cm path length for each of the two compartments [from T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.* **237**, 2481 (1962)].

The construction and use of double cells employed in solvent perturbation studies are shown in Fig. 8. Proper solvent compensation is achieved by filling the front part of the cell compartment containing the protein (or model compound) plus the perturbant, say 20% sucrose, with water or buffer; this is balanced by placing the 20% perturbant in water in the "blank" compartment of the reference cell. The solution compartment of this cell is reserved for the protein solution (or model compound). Customarily the filled cells are positioned in the instrument with the blank compartments facing the source of radiation, so that the light beams enter the blank-containing sections of the cells first.

With the start of a new set of measurements the instrument, with the cells in position, is adjusted to give a satisfactory base line or "zero

line" in the wavelength region of interest. This is most conveniently achieved by use of instruments equipped with a set of potentiometers called "multipots," which permit the base line to be adjusted as a function of wavelength. The use of multipots alleviates the need for correcting the experimentally derived difference spectrum for base-line variation with wavelength. For base-line adjustment both the solvent and solution compartments are filled with "blanks"; that is, solvent is placed in the blank compartment and 20% perturbant in the solution compartment of the sample cell, while the reference cell is filled with the same solutions in reverse order. After the adjustment of the base line the blank compartments of the two cells are tightly stoppered and the solution compartments are rinsed, dried with a jet of air or nitrogen, and finally filled with the test solutions. The same procedure is employed with urea solutions or in experiments involving other organic solvents.¹⁶ The blanks of course in this latter case are the organic solvent and the same solvent containing 20% perturbant, respectively.

Matched cells are selected on the basis of their absorbance differences in the wavelength region of interest. They should be checked with protein solutions, or solutions of tyrosine or tryptophan having an absorbancy value of 2-3, in the individual cell compartments; a scan is made after adjustment of the base line to zero with solvents. Well-matched cells should not produce deviations from the base line in the strongly absorbing region of the spectrum of more than ± 0.002 absorbance unit.

For measurements at low or elevated temperatures, Holmes and Kronman⁷³ have designed a demountable, thermostated, double cell.

Difference spectral measurements have also been made in Beckman DU spectrophotometers and other single-beam instruments. However, because of the problem of monochromatization and stray light errors attending work on optically dense solutions in single-beam instruments,⁷⁴⁻⁷⁶ these measurements are necessarily less precise, as well as more cumbersome. Measurements are usually made in single cells, and the contributions of solvent and perturbant have to be separately determined and subtracted from the uncorrected difference spectrum. In principle, of course, it should also be possible to construct double cells for these instruments, which would eliminate the necessity for the latter measurements.

A simple test for the absence of stray light and monochromatization

⁷³ L. G. Holmes and M. J. Kronman, *Anal. Biochem.* **7**, 124 (1964).

⁷⁴ K. S. Gibson, *U. S. Natl. Bur. Std. Circ.* **484**, (1949).

⁷⁵ L. S. Goldring, R. C. Hawes, G. H. Hare, A. O. Beckman, and M. E. Stickney, *Anal. Chem.* **25**, 869 (1953).

⁷⁶ I. Fridovich, W. Farkas, G. W. Schwert, and P. Handler, *Science* **125**, 1141 (1957).

errors is the adherence of the test solution to Beer's Law (i.e., the plot of the difference spectrum at the two characteristic maxima against concentration is linear). For these reasons it is recommended that a new instrument be calibrated with well-documented test substances such as tyrosine or tryptophan^{13, 30, 46} and that difference spectral measurements be confined to concentrations at which Beer's Law is obeyed. It has been the author's experience, with the Cary Model 14 double-beam instrument, that at absorbancy values below 3 these requirements are adequately met by most protein and model solutions. Limitations of single-beam instruments restrict measurements to lower concentrations, at absorbancies of 2 or less than 2.⁷⁷

Preparation of Solutions for Solvent Perturbation Studies. Exact volumetric manipulations are of paramount importance in difference spectral work. For this reason it is mandatory that errors inherent in pipetting, volumetric adjustment, and transfer of solutions be appreciated and reduced to a minimum.

Herskovits and Laskowski^{7, 16} have presented detailed procedures for preparation of solutions for difference spectral work. Their procedure is abstracted below.

Solutions are diluted serially from clarified stock solutions. pH and ionic strength adjustments are made by mixing 3.0 or 4.0 ml stock solution with 2.0 or 1.0 ml buffer, acid salt, or alkali salt. After mixing, two volumetric deliveries are made, the first to 2.0 ml water and a second to 2.0 ml of the particular 40% perturbant employed for the experiment (sucrose, glycerol, ethylene glycol, etc.). The water and perturbant solutions are kept in stoppered flasks. To minimize drainage and dilution errors due to the viscosity of the solutions delivered, the same pipettes are employed throughout the experiment. Perturbants are delivered first; this is followed by rinsing the pipette and delivery of the aqueous diluent. The same pipette is used to deliver the protein solutions; this can be done by drying the pipette or rinsing it with excess protein solution. In this procedure the content of the tip of the pipette is carefully blown into the flask until no further drainage is observed. Since all the protein solutions of a series are of the same concentration, use of the same pipette minimizes dilution errors. To avoid small changes in pH due to protein adhering to the inner wall of the pipette, the protein solution should be taken up once or twice before each delivery and allowed to drain freely back into the beaker.

Difference spectra should be obtained within 10-30 minutes after

⁷⁷ Deviation from Beer's Law due to high absorbancy of solutions may also be accompanied by distortions in shape of difference spectra. Such spectral changes may assume the form of shifts in the maxima, or even the appearance of artificial maxima or minima.⁷⁸

preparation of solutions. Prolonged standing of solutions has in some cases been found to cause aggregation and attendant turbidity. Turbidity differences can produce a positive or negative slope in the difference spectrum in the nonabsorbing region from 350 to 310 $m\mu$ (see Fig. 2; note that the slope here is zero). In principle, it should be possible to extrapolate the linear portion of the difference spectrum into the absorbing region, and to subtract or add this contribution to the difference spectrum. However, corrections greater than 10% are risky and should not be made. No attempts should be made to clarify these solutions, since the removal of aggregated protein is bound to produce a mismatch in concentrations of the two experimental solutions. However, clarification of the stock solutions by centrifugation or filtration is strongly recommended.

The pH values of both the aqueous and perturbant solutions are recorded after the different spectral run. With freshly prepared perturbant solutions the pH of unbuffered solutions has not been found to differ by more than 0.1 pH unit.⁷

Urea solutions are prepared in a similar manner. Because of the fairly viscous nature of urea solutions containing 40% perturbant, all volumetric manipulations have been performed at approximately 35°. The diluents in these cases are 8 *M* urea and the particular 40% perturbant in the presence of 8 *M* urea. With liquid perturbants it is often more convenient to prepare urea solutions directly in matched volumetric flasks, using the pure additive as diluent. In the latter procedure, 4 volumes of stock solution in 10 *M* urea (at the desired pH and ionic strength) are diluted with 1 volume of pure perturbant, and another 4 volumes of the same solution are diluted with 1 volume of water. A similar procedure has been employed for the preparation of protein solutions in organic solvents such as ethylene glycol, acidic methanol, or 2-chloroethanol.¹⁶

Measurements on disulfide-cleaved proteins, required for the establishment of reference values of $\Delta\epsilon_{\lambda, \max}/\epsilon_{\lambda, \max}$ may be conveniently made in the presence of reducing agent in 8 *M* urea. For such experiments reduction is carried out essentially according to the method of Sela, White, and Anfinsen,^{78, 79} in 10 *M* urea, under nitrogen, using unbuffered 0.05 *M* thioglycolate as reducing agent.⁷ After 4 hours the ionic strength and pH are adjusted, as described above or by addition of appropriate amounts of acidic 10 *M* urea and 10 *M* urea-salt solutions. Reduction may also be carried out with mercaptoethanol or sodium borohydride. Hayashi and co-workers⁴³ have studied the difference spectra of reduced lysozyme

⁷⁸ M. Sela, F. H. White, Jr., and C. B. Anfinsen, *Science* **125**, 691 (1957).

⁷⁹ M. Sela, F. H. White, Jr., and C. B. Anfinsen, *Biochim. Biophys. Acta* **31**, 417 (1959).

in 8 *M* urea in the presence of sodium borohydride (reduced at pH 10 in 30 minutes at 60° with 8 mg/ml of borohydride). Protein cleaved by *S*-sulfonation with sodium sulfite in the presence of cupric ion¹⁵ may also be employed (cf. this volume [22]).

If reducing agent is present, the contribution to direct and difference spectra must be taken into account. Herskovits and Laskowski⁷ found that a 2–3% correction had to be applied to the absorbance of serum albumins at 278 m μ . However, no correction was necessary for the difference spectra, since it was found that 0.02 *M* thioglycolate does not contribute to the difference spectra in the region above 270 m μ .

Solutions for Acid, Alkali, and Temperature Difference Spectra. Dilutions for acid difference spectral studies, or studies exploring the effect of base on the difference spectra of proteins, are usually prepared from a common stock solution in matched 5- or 10-ml volumetric flasks. A set of solutions for a series of spectrophotometric scans consists of a common reference solution and several sample solutions at different pH values but of the same concentration. The reference solution may be prepared by dilution with neutral salt or buffer, whereas the sample solutions are prepared by use of appropriate quantities of acid, base, or buffer to give the desired pH and ionic strength.

Difference spectra produced by temperature changes are studied with solutions of identical concentration, pH, and ionic strength. In such experiments the reference solution is usually maintained at room temperature while the temperature of the sample solution is varied. This may be accomplished by circulating water from a constant temperature bath through the walls of the cell housing or through the outer jacket of specially designed, jacketed cells.^{62, 73} For precise measurements the effects of volume changes with temperature should be taken into account.

Test for Reversibility. The interpretation of difference spectral data in thermodynamic terms requires that the denaturation process studied be reversible. For this reason it is necessary to check the reversibility of temperature-induced difference spectral changes as well as spectrophotometric titration data. In the former case this can be accomplished simply by cooling the sample solution after equilibration at temperatures above the denaturation transition temperature.^{62, 63} In the latter case, solutions may be back-titrated from alkaline pH by use of appropriate quantities of acid or buffer.^{55, 58}